

WO 91/18095(1)  
C12N15/10-

PROPERTY ORGANIZATION  
nal Bureau



- 1 - \* -

**C12N15/10** UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : <b>C12N 15/10</b>		<b>A1</b>	(11) International Publication Number: <b>WO 91/18095</b>
			(43) International Publication Date: 28 November 1991 (28.11.91)
(21) International Application Number: <b>PCT/US91/03382</b>		(74) Agent: PATTERSON, Melinda, L.; Arnold, White & Durkee, P.O. Box 4433, 750 Bering Drive, Houston, TX 77210 (US).	
(22) International Filing Date: 15 May 1991 (15.05.91)			
(30) Priority data: 523,766 15 May 1990 (15.05.90) <b>US</b>		(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), LU (European patent), NL (European patent), SE (European patent).	
(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).			
(72) Inventors: SERWER, Philip ; 9919 Tiaga, San Antonio, TX 78230 (US). LOUIE, Donna, F. ; 7458 Louis Pasteur #106, San Antonio, TX 78229 (US).		<p><b>Published</b></p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: HIGH MOLECULAR WEIGHT DNA COMPOSITIONS FOR USE IN ELECTROPHORESIS OF LARGE NUCLEIC ACIDS

(57) Abstract

The invention provides high molecular weight DNA length standards useful for sizing very long DNA molecules and methods for producing such standards. In a preferred embodiment, the invention provides a mixture of T4 DNA concatemers that forms a DNA "ladder" having rungs differing by at least 0.17 Mb (Megabases) in length upon gel electrophoresis and methods for producing and using such compositions.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

-1-

5 HIGH MOLECULAR WEIGHT DNA COMPOSITIONS  
FOR USE IN ELECTROPHORESIS OF LARGE NUCLEIC ACIDS

10 FUNDING: Development of the present invention was aided  
in part by funding from The National Institutes of Health  
(Grant no. GM24365). Accordingly, the Federal Government  
may own certain rights. Development of the invention was  
also facilitated by funding from the Texas Coord. Board  
(Grant No. 004).

15 The present invention provides high molecular weight  
DNA length standards useful for sizing very long DNA  
molecules and methods for producing such standards. In a  
preferred embodiment, the invention provides a mixture of  
20 T4 DNA concatemers (end-to-end multimers of the monomeric  
DNA) that forms a DNA "ladder" having rungs differing by  
at least 0.17 Megabases (Mb) in length upon gel  
electrophoresis and methods for producing and using such  
compositions. Also included are compositions containing  
25 pentameric or hexameric concatemers of T4 DNA and methods  
for blunt-end ligation of DNA to produce extremely long  
(e.g. 1 Mb) DNA molecules.

30 As the study of molecular biology has evolved,  
workers in the field have strived to manipulate and  
fractionate by size larger and larger pieces of DNA,  
using the molecular tools previously successful with  
smaller DNA fragments. However, serious difficulties  
arise when manipulating very large DNA molecules. Recent  
35 advances in the field of electrophoresis utilize rotating  
gels or pulsing electric fields. Fortunately, using  
these new techniques, it is now possible to fractionate  
by length DNA molecules as long as 10 Mb (Cantor, et al.,  
1988; Cantor and Schwartz, 1984; Serwer, 1987; U.S.

Patent Application, Serial No. 393,084 incorporated herein by reference).

5 One of the major problems remaining for workers wishing to use the new techniques is the lack of stable, reproducible, discrete length standards. The most useful primary length standards used in the past have been obtained by annealing single-stranded cohesive ends of mature, 49 Kilobase (kb) bacteriophage  $\lambda$  DNA to form  
10 concatemers that produce a series of bands or "ladder" during electrophoresis.

Unfortunately, the relatively short size of the  $\lambda$  DNA makes it poorly suited for use as standards in sizing very long ( $\geq 1.5$  Mb) DNAs. Further, concatemerization of  $\lambda$  DNA requires 12 bp cohesive terminal repeats which may be damaged before or during the concatemerization process, thereby further limiting the length of the concatemers. The  $\lambda$  concatemers that do form are not  
20 particularly stable to denaturation since the overlapping ends are quite short. Thus, when used under conditions that even mildly denature DNA, such as elevated temperature, bacteriophage  $\lambda$  standards are destabilized and rendered useless as DNA length markers.

25 Additional problems arise with the  $\lambda$  standards if the standard preparation is allowed to sit for even short periods of time. Since the concatemerization is non-enzymatic, the preparations tend to further concatemerize over time giving rise to non-reproducible results from  
30 one usage to the next and to a much shorter shelf life.

An improved DNA sizing ladder, obtainable by a process employing enzymatic concatemerization of the so  
35 called "T-odd" bacteriophage (particularly T7) DNA (40 kb), was described in copending patent application no. 293,235 and incorporated herein by reference. However,

-3-

that method involved use of a relatively complex mixture, involving cellular enzymes and extracts and the like. Thus, although the methods represented a great advance in the art, significant room for improvement remained, and compositions of DNA for sizing very long DNA molecules continued to be sought.

Fortunately, the present inventors have now discovered a convenient, reproducible technique for generating stable DNA compositions suitable for sizing very long DNA molecules. The technique has allowed production of DNA "ladders" having rungs spaced by more than 0.05 Mb, and has even allowed production of ladders with at least 6 rungs spaced by up to 0.17 Mb. The method has been successfully employed to join blunt ended segments of DNA to produce very long molecules (greater than about 1 Mb).

One embodiment of the invention includes a composition of matter comprising a first DNA species of about 1x Mb in length, a second DNA species of about 2x Mb in length, and a third DNA species of about 3x Mb in length. In this embodiment, x is a constant greater than about 0.05. When subjected to gel electrophoresis, the composition is capable of generating a series of discrete bands, the DNA within any one of such bands differing in length from the DNA of any other one of such bands by an integral multiple of x. In yet a further embodiment, a composition of matter is provided that consists essentially of a first DNA species of about x Mb in length, a second DNA species of about 2x Mb in length, and a third DNA species of about 3x Mb in length, wherein x is a constant greater than about 0.05, and more preferably, is at least about 0.17.

Also included is a composition of matter comprising a mixture of monomers and oligomers of a selected DNA

-4-

molecule. In this embodiment the monomers of the molecule are at least about  $x$  Mb in length. Preferably,  $x$  is greater than about 0.05 Mb, and more preferably  $x$  is about 0.17. Further, the mixture is characterized in that, upon electrophoresis under suitable conditions, the DNA migrates to form discrete bands of DNA, each band containing a species of DNA of about  $n(x)$  Mb in length, wherein  $n$  is an integer. Preferably, the monomer DNA species will be a "blunt ended" species. In a more preferred embodiment the oligomers will comprise dimers, and trimers, and more preferably, tetramers. In an even more highly preferred embodiment, the oligomers will comprise pentamers, or even more preferably, hexamers. In yet a further embodiment, the selected DNA molecule of the composition is a T4 bacteriophage DNA of about 0.17 Mb in length (as a monomer).

The invention also includes a number of novel methods for preparation of the compositions. For example, the invention includes a method for preparing a mixture of monomers and oligomers suitable for use as DNA size standards comprising the following steps: obtaining a purified preparation of a selected DNA molecule of at least about  $x$  Mb in length (wherein  $x$  is a constant greater than about 0.05), mixing the preparation with a ligase and polyethylene glycol under conditions suitable to allow a concatemerization reaction to occur; and terminating the concatemerization reaction to obtain a stable mixture comprising the DNA molecules and concatemers thereof. In an embodiment that is more highly preferred, termination of the concatemerization produces a stable mixture containing concatemers of the DNA molecules, characterized in that, upon electrophoresis under suitable conditions, the DNA molecules migrate to form a series of discrete bands of DNA, the DNA in each band differing in length from the DNA in any other band by about  $n(x)$  Mb, wherein  $n$  is an

-5-

integer. Preferably, the DNA molecules are blunt ended, and more preferably T4 molecules and x is about 0.17. T4 ligase is most advantageously used. The polyethylene glycol will generally comprise a polyethylene glycol of high molecular weight, for example, at least about 3000, and most preferably at least about 6000.

The invention also includes a method for joining large blunt-ended pieces of DNA to produce ligated DNA molecules of at least about 1 Mb in length. Such ligated molecules are advantageous in both size and stability. Unlike molecules joined by complementary base pairing of so-called "cohesive ends," blunt end ligated molecules are not subject to depolymerization under conditions (such as increased temperature or changes in salt concentration) which can cause the cohesive ends to "melt" or come apart. According to the invention, therefore, there is provided a method for blunt end ligation of DNA which comprises the steps of obtaining a preparation of blunt ended DNA molecules; incubating the preparation together with a ligase and polyethylene glycol under conditions suitable for ligation of the blunt ended DNA molecules; and terminating the ligation reaction to obtain a preparation comprising DNA molecules of at least 1 Mb in length.

Although certain of the reaction conditions for concatemerization may be varied, the present inventors have discovered a unique set of reaction conditions that are exceedingly and surprisingly satisfactory. The invention includes a method wherein the concatemerization conditions comprise about 20  $\mu\text{g/ml}$  DNA, more preferably about 18.75  $\mu\text{g/ml}$ ; about 10% (w/w) polyethylene glycol 6000 or more preferably 9.5% (w/w); about 70 mM NaCl, more preferably 67.5 mM; about 7 mM  $\text{NaPO}_4$ , pH 7.4, more preferably, 6.75 mM; about 0.7 mM EDTA, pH 7.4, more preferably 0.675 mM; in a buffer comprising about 50 mM

-6-

Tris-Cl, pH 7.4 about 10 mM  $MgCl_2$ , about 20 mM dithiothreitol, about 1 mM ATP, and about 50  $\mu g/ml$  bovine serum albumin. Preferably, the termination step will be performed after between about 5 hours and about 10 hours of incubation, and most preferably after about 6 hours of incubation. Also preferred is a method wherein the termination step comprises inactivating the ligase by heating, for example, at about 75°C for about 15 minutes.

Using these methods, the present inventors have achieved ladders of DNA with rungs many times further apart than ever before achieved. Accordingly, the present invention also includes a T4 DNA concatemer of at least about 1 Mb in length, and a pentamer or a hexamer of a 0.17 Mb T4 DNA molecule.

These and other aspects of the invention will become more apparent from a description of particular embodiments when read in conjunction with the drawings.

Figure 1: Panel (a) depicts T4 DNA ladders; Panel (b) depicts T7 DNA ladders. The gels were stained with ethidium bromide. The length of DNA in Mb is indicated.

In a general and overall sense, the present invention includes the steps of preparing a concatemer (or oligomer) of a selected DNA monomer, where the selected monomer comprises more than about 0.05 Mb. Although a number of suitably long DNA monomers may be successfully employed with the aid of this disclosure, preferably, the monomer will comprise a long blunt ended segment of DNA such as mature T4 DNA or DNA from bacteriophage G or T5 (0.12 Mb). Although the DNA will usually be double stranded, when the standard is desired for use in sizing a single stranded DNA preparation, use of a single stranded concatemer may be preferred. The DNA may be extracted from the bacteriophage by any of a



-7-

number of suitable methods, for example, those described below in example I, taking care to avoid unnecessary breakage of the DNA molecules during the isolation procedure. The invention also provides a method whereby  
5 long segments of blunt ended DNA can be ligated to produce very long (1 Mb or more) DNA molecules.

For the reaction, the DNA is incubated in a suitable incubation mixture for between about 3-6 hours, and most  
10 preferably about 6 hours. Generally, the mixture will contain a high molecular weight polyethylene glycol such as polyethylene glycol 4000, 6000, or 12500, a DNA ligase (preferably T4 ligase), and a suitable reaction buffer. Most commonly, the PEG concentration will comprise about  
15 10% by weight of the final ligation mixture. Although incubation at 37°C is preferred, the incubation step may also be conducted at room temperature. After incubation, the concatemerization reaction is terminated by an inactivation of the ligase, preferably by heating, e.g.,  
20 at about 75°C for 15 minutes. Less satisfactory results are obtained when one uses 0.01 M EDTA to stop the reaction.

These and other aspects of the invention will become  
25 more apparent when read in context of the examples below. The examples are not intended to limit the scope of the claims unless so specified in the claims therein.

#### A. Preparation of Bacteriophage Stock

30 A preparation of T4 bacteriophage was added to a lawn of log phase *E. Coli* BB/1 on T broth plates. The plates were incubated at 30°C for approximately 24-30 hours to allow formation of plaques. A plate containing approximately 25-100 plaques was selected, a single  
35 isolated plaque was selected, and a plug of agar containing the plaque (bacteriophage plug) was removed from the plate, and transferred to sterile T7 buffer (0.5

-8-

M NaCl, 0.01 M Tris-Cl, pH 7.4, .001 M  $MgCl_2$ , 1 mg/ml gelatin) containing a few drops of chloroform. The bacteriophage plug was stored in this solution at 4°C for up to approximately one week.

5

In the next step of the procedure, a log phase culture of BB/1 was grown to a density of approximately  $1 \times 10^8$  cells per ml in 2X LB (Luria-Bertani) medium. Then, a bacteriophage plug, prepared as described above, was added to 100 ml of the BB/1 culture. After incubation with the bacteriophage for about four hours, the bacteria were lysed by addition of chloroform. For that procedure, several drops of chloroform were bubbled into the mixture until the bacteria lysed. At the same time that the chloroform was added, NaCl was added to a final concentration of 1 M. Bacterial debris was removed by centrifugation at 7000 RPM for 7 minutes. After centrifugation, the supernatant, which contained the T4 bacteriophage, was removed. The titer of the bacteriophage stock was determined by plating the bacteriophage on a lawn of *E. Coli* BB/1.

The titered bacteriophage stock was used in a subsequent large scale infection to produce T4 bacteriophage from which T4 DNA was obtained. For that procedure, an isolated colony of *E. coli* BB/1 was inoculated into a 50 ml flask of medium M9. After overnight incubation, a 25 ml aliquot of the initial preparation was added to 6 liters of M9 medium and the mixture was cultured to a bacterial cell density of  $3 \times 10^8$  cells per ml. The bacteria were infected with T4 bacteriophage at a multiplicity of infection of  $10^{-3}$ , and the mixture was incubated for about 3 hours. Then, the culture was brought to 0.5 M sodium chloride, 22 ml of chloroform, and 8-10% polyethylene glycol 6000 was added to the preparation. The mixture was maintained at 4°C for about 2-7 days. Then, the mixture was centrifuged at

-9-

4°C for 45 min at 4000 RPM to pellet the bacteriophage. The sedimented material, i.e., the bacteriophage, was resuspended in approximately 130 ml of sterile T7 buffer without gelatin, carbowax was added to 9%, and the mixture was incubated at 4°C overnight. The material was centrifuged at 5000 RPM for 10 min, the pellet was resuspended in 5.5 ml T7 buffer without gelatin containing a final concentration of 2.5 µg/ml DNase I and incubated at 30°C for one hour.

A cesium chloride (0.01 M Tris-Cl, pH 7.4, 0.001 M. MgCl<sub>2</sub>) step gradient was prepared as follows:

	<u>n<sup>25</sup></u>	<u>Vol.</u>	
Top	1.3550	1.5 ml	η - refractive index
	1.3610	1.0 ml	
	1.3760	1.5 ml	
	1.3820	1.0 ml	
Bottom	1.4020	1.0 ml	

The bacteriophage was purified further by centrifugation over the cesium chloride step gradient at 18°C for three hours using an SW41 rotor and a speed of 33,000 RPM. After centrifugation, the bacteriophage were compressed into a visible band, which sedimented at a refractive index of about 1.3810. The band was removed from the tube and adjusted to a refractive index of 1.3810 in cesium chloride 0.01M Tris-Cl, pH 7.4, 0.001M MgCl<sub>2</sub>. Then, the suspension was centrifuged on a cesium chloride buoyant density gradient at 40,000 rpm for 20 hours at 10°C in an SW50.1 rotor, and the bacteriophage-containing band was collected as before. The cesium chloride was removed from the bacteriophage suspension by successive dialysis through a series of buffers containing decreasing salt concentrations as follows:

-10-

	NaCl	Tris-Cl (pH 7.4)	MgCl <sub>2</sub>
	2.5M	0.01M	0.001M
	2.0M	0.01M	0.001M
5	1.25M	0.01M	0.001M
	1.0M	0.01M	0.001M
	0.05M	0.01M	0.001M
	0.1M	0.01M	0.001M
	0.2M	0.01M	0.001M

10

#### Phenol Extraction of DNA

T4 DNA was then purified from the bacteriophage preparation by phenol extraction. For that procedure, T4 bacteriophage was diluted in TE buffer (0.01M Tris-Cl, pH 7.4, 0.001M EDTA) to an OD<sub>260</sub> of approximately 50. A 150 microliter aliquot of the diluted bacteriophage was added to an equal volume of phenol equilibrated in TE buffer in a 1.5 ml conical microfuge tube and mixed by gently inverting the tube for about 100 seconds. After that time, the mixture was centrifuged for 5 minutes in a microfuge at room temperature. The upper aqueous layer of the supernatant was transferred to a new 1.5 ml tube, an equal volume of phenol was added, and the mixture was mixed by gently inverting the tube for approximately 100 seconds. The centrifugation step was repeated, and the upper aqueous layer was removed and mixed with an equal volume of 1:1 phenol:chloroform-isoamyl alcohol (24:1). The mixture was centrifuged for 5 minutes in a microfuge at room temperature. The upper aqueous layer of the resultant supernatant was removed and dialyzed against sterile buffer (0.1M NaCl, 0.01M Tris-Cl, pH 7.4 and 0.001M EDTA) with stirring and three changes at 4°C overnight. After dialysis, the DNA concentration was measured by a spectrophotometer at OD<sub>260</sub> and stored at 4°C.

#### Concatemerization and Electrophoresis

The T4 DNA prepared above comprised blunt-ended double stranded DNA monomers of about 0.17 Kb in length.

-11-

5 The DNA monomers were concatemerized to form oligomers capable of forming a DNA ladder with 0.17 Mb rungs on electrophoresis as follows: Three  $\mu$ l of 250  $\mu$ g/ml DNA were added to a mixture containing: (a) 27  $\mu$ l of 14% (w/w) polyethylene glycol (PEG) 6000 in 0.1 M sodium chloride, 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA, (b) 8  $\mu$ l of 0.25 M Tris-Cl, pH 7.4, 0.05 M  $MgCl_2$ , 0.1 M dithiothreitol (DTT), 0.005 M ATP, 250  $\mu$ g/ml bovine serum albumin (BSA), and (c) 1  $\mu$ l of 3.3 units/ $\mu$ l T4 ligase (U.S. Biochemicals). Thus, the final concentration of reagents in the mixture was as follows: 18.75  $\mu$ g/ml DNA; 9.5% PEG 6000, 67.5 mM NaCl, 6.75 mM  $NaPO_4$ ; 0.675 mM EDTA. The ligation buffer contains 50 mM Tris-Cl, pH 7.4, 10 mM  $MgCl_2$ ; 20 mM DTT, 1 mM ATP and 50  $\mu$ g/ml BSA.

15 After incubation at 37°C for 6 hr, the ligase was inactivated by incubation at 75°C for 15 min to terminate the concatemerization reaction. Twenty-five  $\mu$ l of the ligation mixture (469 ng) was loaded into a well of 1% Seakem LE agarose gel cast in 0.01 M sodium phosphate, pH 7.4 and 0.001 M EDTA. The gel was placed in a rotating gel electrophoresis apparatus (see U.S. Patent Application Serial No. 393,084, which is expressly incorporated herein by reference) and was subjected to electrophoresis at a pulse time of 120 seconds and an angle of rotation at  $1.4\pi$  radians. A DNA ladder with 0.17 Mb rungs was observed. The length of the ladder appeared to be limited by the amount of broken DNA in the ligation mixture. (Fig. 1a, lane 1). The T4 ladder was not formed when the PEG was omitted (Fig. 1a, lane 2). When the ligation of Fig. 1a, lane 1 was performed using the conditions described above by use of 0.04 Mb T7 DNA, and a 11-12 rung ladder was observed (Fig. 1b).

35 The foregoing description of the invention has been directed to particular preferred embodiments in accordance with the requirements of the patent statutes

-12-

and for purposes of explanation and illustration. It will be apparent, however, to those skilled in the art that many modifications and changes may be made without departing from the scope and the spirit of the invention.

-13-

Claims:

1. A composition of matter comprising:

- 5 a first DNA species of about  $x$  Mb in length;  
a second DNA species of about  $2x$  Mb in length; and  
10 a third DNA species of about  $3x$  Mb in length.

Wherein  $x$  is a constant greater than about 0.05, and  
wherein the composition is capable of generating a  
series of discrete bands when subjected to gel  
15 electrophoresis wherein the DNA in any one of such  
bands differs in length from the DNA in any other of  
the band by an integral multiple of about  $x$ .

2. A composition of matter consisting essentially of:

- 20 a first DNA species of about  $x$  Mb in length;  
a second DNA species of about  $2x$  Mb in length; and  
25 a third DNA species of about  $3x$  Mb in length;  
wherein  $x$  is a constant greater than about 0.05.

30 3. The composition of claim 1 or claim 2 wherein  $x$  is  
greater than or equal to about 0.17.

35 4. A mixture of T4 DNA and oligomers thereof, wherein  
when the mixture is subjected to electrophoresis under  
suitable conditions, the DNA migrates to form discrete  
bands of DNA, each of the bands containing a species of

-14-

DNA of about  $n(0.17)$  Mb in length, and wherein  $n$  is an integer.

- 5      5. A composition of matter comprising a mixture of monomers and oligomers of a selected DNA molecule, and wherein the monomers of the molecule are at least about  $x$  Mb in length and  $x \geq$  about 0.05.
- 10      6. The composition of claim 5 wherein  $x$  comprises at least about 0.17.
- 15      7. The composition of claim 5 wherein the DNA molecule comprises a T4 DNA.
- 20      8. The composition of claim 5 wherein oligomer includes dimers, and trimers.
- 25      9. The composition of claim 5 wherein oligomer includes tetramers.
- 30      10. The composition of claim 5 wherein oligomer includes pentamers.
- 35      11. The composition of claim 5 wherein oligomer includes hexamers.
12. The composition of claim 5 wherein when the mixture is subjected to electrophoresis under suitable conditions, the DNA migrates to form discrete bands of



-15-

DNA, each band containing DNA of about  $n(x)$  Mb in length, wherein  $n$  is an integer.

- 5        13. A T4 concatemer of at least about 1 Mb in length.
- 10       14. An oligomer of a selected DNA monomer wherein the oligomer comprises at least about five units of the monomer and the monomer is at least about 0.17 Mb in length.
- 15       15. A pentamer of a monomer of mature blunt ended T4 DNA.
- 20       16. A hexamer of a monomer of mature blunt-ended T4 DNA.
- 25       17. A method for preparing a mixture of monomers and oligomers suitable for use as a DNA size standard comprising:
- 30           obtaining a purified preparation of a selected DNA molecule of at least about 0.05 Mb in length;
- mixing the preparation with a ligase and polyethylene glycol under conditions suitable to allow a concatemerization reaction to occur; and
- 35           terminating the concatemerization reaction to obtain a stable mixture comprising the DNA molecules and oligomers thereof.

-16-

18. A method for preparing a mixture of DNA molecules suitable for use as a size standards for gel electrophoresis comprising the steps of:

5 obtaining a preparation of DNA molecules of a length  $x$ , wherein  $x$  is a constant greater than about 0.05 Mb;

10 incubating the preparation together with a ligase and polyethylene glycol under conditions suitable for concatemerization of the molecules to occur;

15 terminating the concatemerization reaction to produce a stable mixture containing oligomers of the DNA molecules, the mixture characterized in that upon electrophoresis under suitable conditions, the DNA molecules migrate to form a series of discrete bands of DNA, the DNA in each band differing in length from the DNA in  
20 any other band by about  $x$  Mb.

25 19. The method of claim 18 wherein the DNA molecules are T4 DNA molecules and  $x$  is 0.17.

30 20. The method of claim 18 wherein the ligase is T4 ligase.

35 21. The method of claim 18 wherein the polyethylene glycol has a molecular weight greater than about 3000.

22. The method of claim 18 wherein the concatemerization conditions comprise:

-17-

about 20  $\mu\text{g/ml}$  DNA;

about 10% polyethylene glycol 6000;

5 about 70 mM NaCl;

about 7 mM  $\text{NaPO}_4$ ;

10 about 0.7 mM EDTA;

in a buffer comprising about 50 mM Tris-Cl, pH 7.4,  
about 10 mM  $\text{MgCl}_2$ ; about 20 mM dithiothreitol, about  
1 mM ATP, and about 50  $\mu\text{g/ml}$  bovine serum albumin.

15

23. The method of claim 18 wherein the termination step  
occurs after between about 5 hours and about 10 hours of  
incubation.

20

24. The method of claim 18 wherein the termination  
occurs after about 6 hours of incubation.

25

25. The method of claim 18 wherein the termination step  
comprises inactivating the ligase by heating.

30

26. The method of claim 18 wherein the termination step  
comprises heating the incubated preparation at  $75^\circ\text{C}$  for  
about 15 minutes.

35

27. A method for blunt-ended ligation of DNA which  
comprises the steps of:

-18-

obtaining a preparation of blunt ended DNA molecules;

5 incubating the preparation together with a ligase and polyethylene glycol under conditions suitable for ligation of the blunt ended DNA molecules to one another;

10 terminating the ligation reaction to obtain a preparation comprising DNA molecules of at least 1 Mb in length.

15 28. The method of claim 27 wherein the ligase is T4 ligase.

20 29. The method of claim 27 wherein the polyethylene glycol has a molecular weight greater than about 3000.

30. The method of claim 27 wherein the ligation conditions comprise:

25 about 20  $\mu\text{g/ml}$  DNA;

about 10% polyethylene glycol 6000;

30 about 70 mM NaCl;

about 7 mM  $\text{NaPO}_4$ ;

about 0.7 mM EDTA;

35 in a buffer comprising about 50 mM Tris-Cl, pH 7.4, about 10 mM  $\text{MgCl}_2$ ; about 20 mM dithiothreitol, about 1 mM ATP, and about 50  $\mu\text{g/ml}$  bovine serum albumin.

-19-

31. The method of claim 27 wherein the termination step occurs after between about 5 hours and about 10 hours of incubation.

5

32. The method of claim 27 wherein the termination occurs after about 6 hours of incubation.

10

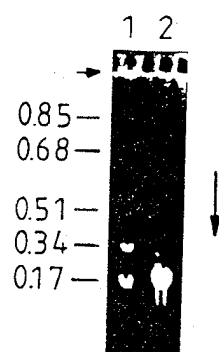
33. The method of claim 27 wherein the termination step comprises inactivating the ligase by heating.

15

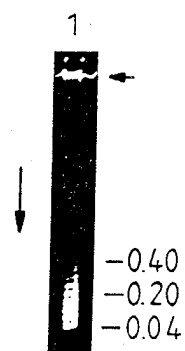
34. The method of claim 27 wherein the termination step comprises heating the incubated preparation at 75°C for about 15 minutes.

1/1

**FIG.1a**



**FIG.1b**



SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 91/03382

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 Int.Cl.5 C 12 N 15/10

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

Int.Cl.5

C 12 N 15/00

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
------------------------	--	-------------------------------------

X	NUCLEIC ACIDS RESEARCH, vol. 12, no. 14, 25 July 1984, G.F. CARLE et al.: "Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis", pages 5647-5664, see the whole article, especially page 5655 ---	1,2,5,8 -12
X	BIOCHEMISTRY, vol. 27, no. 26, 27 December 1988, American Chemical Society, M.K. MATHEW et al.: "High-resolution separation and accurate size determination in pulsed-field gel electrophoresis of DNA. 1. DNA size standards and the effect of agarose and temperature", pages 9204-9210, see the whole article --- <div style="text-align: center;">-/-</div>	1,2,5,8 -12

<sup>10</sup> Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

12-09-1991

Date of Mailing of this International Search Report

- 3. 10. 91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

M. PEIS

M. Peis

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	NUCLEIC ACIDS RESEARCH, vol. 17, no. 13, July 1989, IRL Press, (Oxford, GB), D.J. HANLON et al.: "Plasmid multimers as high resolution molecular weight standards for pulsed field gel electrophoresis", page 5413, see the whole article	1, 17, 27
P, X	--- NUCLEIC ACIDS RESEARCH, vol. 18, no. 20, 25 October 1990, IRL Press, (Oxford, GB), J.C. WHITLEY et al.: "Ligated mycoplasma genomes as DNA size markers for PFGE", pages 6167-6168, see the whole article	1-3, 5, 6 , 8-12, 14, 17, 18, 20, 21, 23, 27-29, 31
P, X	--- WO, A, 9012891 (GENMAP ICN.) 1 November 1990, see pages 9, 28-29 -----	1, 2, 5, 8 -12, 14



US 9103382  
SA 48376

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/09/91  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9012891	01-11-90	AU-A- 3730689	16-11-90